A systematic review of *in vitro* cytokine production in eating disorders

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Abstract

Background: Eating disorders (EDs) have been associated with alterations in cytokine concentrations and production. This review examines whether *in vitro* cytokine production (i) is altered in people with EDs compared to healthy participants; and (ii) changes in response to treatment?

Methods: Using PRISMA guidelines, we systematically reviewed articles reporting group comparisons or longitudinal assessments of spontaneous and/or stimulated cytokine production *in vitro* in EDs.

Results: Twelve studies were included. Cross-sectional results were mixed in anorexia nervosa (AN). Only one study measured cytokine production in bulimia nervosa. Two longitudinal studies showed that daily yoghurt consumption increases phytohemagglutinin-stimulated interferon-γ production in AN.

Conclusion: The mixed results could be accounted for by variations in experimental design. Our findings suggest that cytokine production could possibly be modulated through dietary interventions. However, due to the methodological heterogeneity and shortcomings of the included studies, it seems unreasonable to draw further conclusions.

Keywords: eating disorders, cytokines, anorexia nervosa, bulimia nervosa

1. Introduction

Eating disorders (EDs), including anorexia nervosa (AN), bulimia nervosa (BN) and binge-eating disorder (BED), are characterised by disturbances in eating behaviours and body image. The aetiology of EDs is complex and the underlying pathophysiological mechanisms contributing to their development and maintenance are unclear. Alterations in immunological function and more specifically, the production of cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, have been implicated as a possible contributing factor. For example, recent meta-analyses have shown elevated levels of certain pro-inflammatory cytokines including TNF-α and interleukin IL-6 (Dalton et al., 2018; Solmi et al., 2015), in people with AN compared to healthy individuals. However, those with BN did not significantly differ in concentrations of pro-inflammatory cytokines in comparison to healthy volunteers and no research in BED was available to be incorporated into the meta-analyses (Dalton et al., 2018).

Cytokines are soluble intercellular signalling proteins that are produced by a range of cells, including microglia and astrocytes, in both the brain and in the periphery (Lichtblau et al., 2013). They have particular importance in the immune system, but also in brain functioning (for a review see Capuron and Miller, 2011). It has been well documented that cytokines are involved in the regulation of appetite and feeding. Multiple cytokines have been shown to have inhibitory effects on food intake (Buchanan and Johnson, 2007; Plata-Salaman, 2001; Wong and Pinkney, 2004). For example, reductions in food intake have been observed following peripheral and/or central administration of interleukin (IL)-1β (Langhans et al., 1993) and tumor necrosis factor (TNF)-α (Bodnar et al., 1989) in animal models. In addition, in a validated animal model of binge-like eating behaviour, in which cycles of restriction are combined with frustration stress, down-regulation of the anorexigenic IL-18 system was observed (Alboni et al., 2017). The impact on appetite and feeding regulation is due to both direct and indirect effects of cytokines, for example, through interactions with orexigenic and anorexigenic neurohormones, neuropeptides, and neurotransmitters (e.g., Amaral et al., 2006; Romanatto et al., 2007; Wang et al., 2006), and effects on the central nervous system and also neurons in the hypothalamus, the 'feeding centre' of the brain (Plata-Salaman et al., 1996) (Holden and Pakula, 1996).

Cytokines have also been shown to play a mediatory role in the complex relationship between the immune and neuroendocrine systems. Administration of cytokines has been shown to activate the hypothalamic pituitary adrenal (HPA) axis, stimulating the expression and release of key hormones, including corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol (Besedovsky and del Rey, 1996; 2007). This is of particular importance in EDs as dysregulation, and more specifically hyperactivation, in the HPA axis has been identified (Lo Sauro et al., 2008; Warren, 2011). Cytokines have also been shown to influence neurotransmitter synthesis, release and reuptake (Capuron and Miller, 2011; Holden and Pakula, 1996). This is of relevance as dysregulation in neurotransmitter systems, such as those related to serotonin and dopamine, have been observed in EDs (Kaye, 2008).

It is also of interest that cytokines have been implicated in the pathophysiology of mental disorders that are highly comorbid with EDs, such as depression (Lichtblau et al., 2013) and anxiety disorders (e.g., generalised anxiety disorders, obsessive compulsive disorder, post-traumatic stress disorder) (Furtado and Katzman, 2015). Therefore, cytokines may be involved in the psychopathology associated with EDs, their development and maintenance (Brown et al., 2008; Corcos et al., 2003; Holden and Pakula, 1996; Marcos, 1997; Nova et al., 2002b; Slotwinska and Slotwinski, 2017).

Cytokine production indicates an inflammatory response in the body and potential causes for cytokine changes in patients with EDs could include infections or a subsequent excessive use of antibiotics, autoimmune or autoinflammatory diseases, and the composition of the gut microbiota, as these factors have been found to be associated with the development of EDs (Breton et al., 2016; Morita et al., 2015; Morris et al., 2016; Raevuori et al., 2016; Zerwas et al., 2017). Recent reviews have focussed on circulating cytokine concentrations *in vivo* (Dalton et al., 2018; Solmi et al., 2015), therefore, there has been no recent collation of the evidence related to cytokine production *in vitro,* even though simply measuring plasma or serum levels of cytokines does not reflect the responsiveness of immune cells

following an immune challenge and thus does not provide an insight into the dynamics of an individual's immune response. The current study aims to address this by conducting a systematic review of cross-sectional and longitudinal studies assessing *in vitro* cytokine production across all EDs. We aim to provide an answer to the following two research questions: (i) is *in vitro* cytokine production altered in people with EDs compared to HCs; and (ii) does *in vitro* cytokine production change longitudinally in response to treatment interventions?

2. Material and methods

This review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009).

2.1. Selection criteria

Studies in English of any design that assessed stimulated or un-stimulated cytokine production *in vitro* with a Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association, 1980; 2000; 2013) or International Classification of Diseases (ICD) (World Health Organization, 1992) diagnosis of an ED were eligible for inclusion. Publications were included if they reported cross-sectional comparisons of cytokine production between ED groups and healthy control (HC) groups or longitudinal assessments.

Studies were excluded if: i) they assessed in vivo cytokine concentrations or genetic expression but did not assess cytokine production *in vitro*; ii) they did not report group comparisons in cytokine production or did not report longitudinal measurements of cytokine production; iii) reported cross-sectional or longitudinal measurements without statistical comparisons between groups or across time points, respectively; iv) the sample was comprised of animals; or v) participants had an organic cause for their disordered eating e.g. cancer, immunological conditions, genetic disorder, other medical illness etc.. Review articles, meta-analyses, conference proceedings/abstracts, editorials, letters, book chapters, and unpublished theses were also not included.

2.2. Search strategy

Three electronic databases (PubMed, ISI Web of Science Core Collection, MEDLINE via Ovid SP) were searched from inception until 10th August 2018, using the following keywords and mapped to subject headings with the explode function where possible: "eating disorder*", "anorexia nervosa", bulimi*, "binge eat*" in combination with cytokine*, inflammat*, interleukin, interferon, IFN, "tumor necrosis factor", TNF. Searches were limited to the English language. These searches were supplemented by internet searches and hand-searches of reference lists of potentially relevant papers and reviews. Citation tracking in Google Scholar was also performed.

Titles and abstracts of retrieved publications were imported into EndNote, duplicates were removed, and papers that were deemed highly unlikely to be relevant were disregarded. Full-text versions of the remaining articles were then obtained and screened according to the pre-specified eligibility criteria described above. All papers that did not meet the inclusion criteria were excluded, with the reasons documented (see Figure 1). The entire search process was conducted independently by two reviewers (B.D. and O.P.) and disagreements at the final stage were resolved by consensus.

2.3. Data extraction

Two reviewers (B.D. and V.W.) extracted data from all included studies into electronic summary tables, which were then checked by another reviewer (O.P.). Information collected related to sample characteristics, parameters of interest, measurement methods, and relevant findings. Given that cytokine measurement is affected by a range of confounding factors (e.g., age, smoking status, medication, concurrent physical or mental illness, menstruation) (Dugué et al., 1996), details regarding these were also collected and are shown in Table 1. Due to the authors' knowledge of the area and a broad initial search, a narrative synthesis of the research was decided on prior to conducting the systematic search, given the methodological diversity of the studies. The search, screening and extraction process are detailed in the Supplementary File.

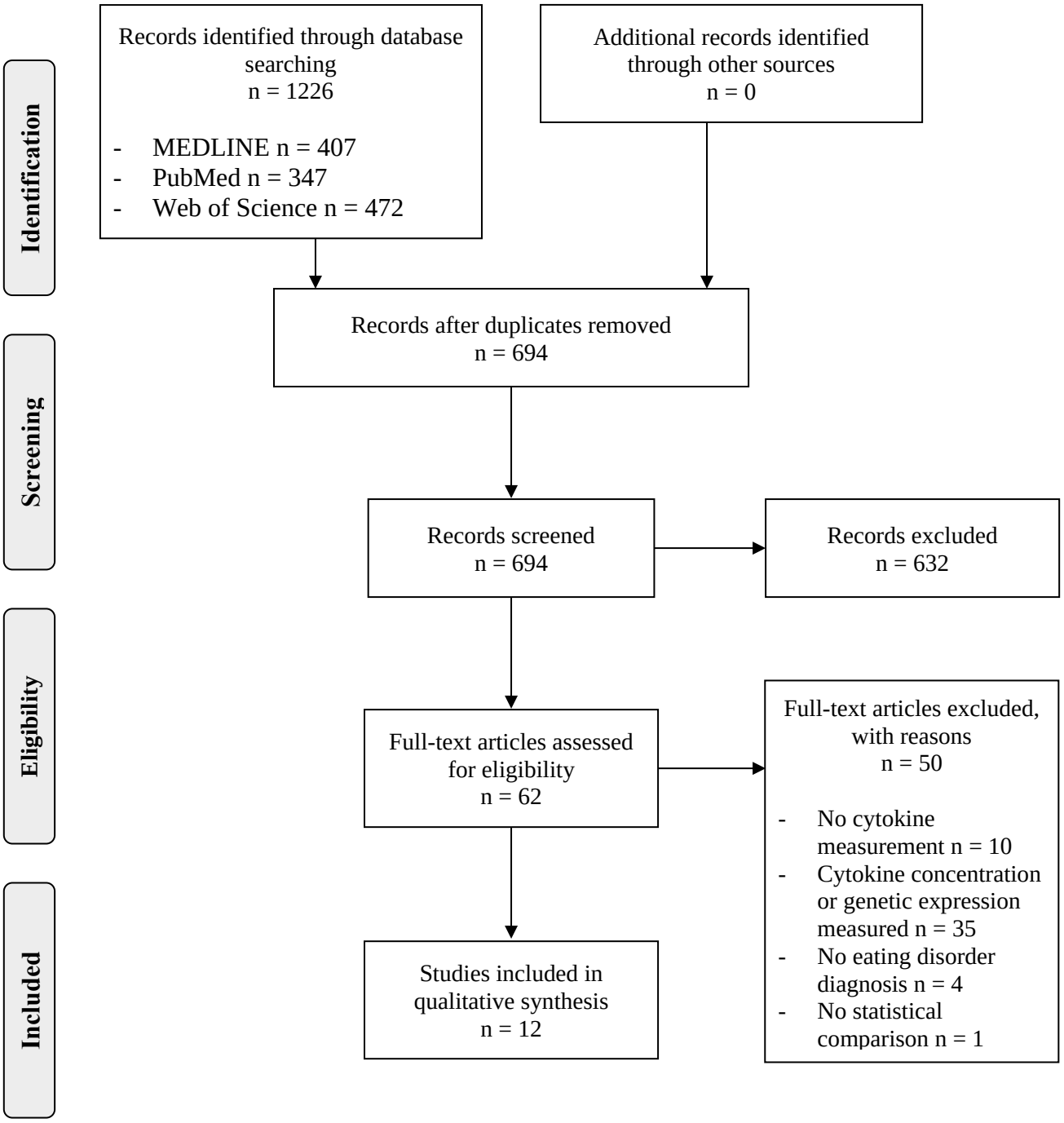

Figure 1. PRISMA flow diagram of search.

3. Results

3.1. Characteristics of Included studies

We identified 12 studies which met the inclusion criteria (see Figure 1 for PRISMA flow diagram), reporting on a total of 271 people with EDs. Nine studies reported cross-sectional comparisons in cytokine production between ED groups and HCs (see Table 2 for cross-sectional study details and findings) (Allende et al., 1998; Bessler et al., 1993; Limone et al., 2000; Nova et al., 2002a; Omodei et al., 2015; Polack et al., 1993; Raymond et al., 2000; Vaisman et al., 1996; Vaisman and Hahn, 1991). Four studies conducted longitudinal assessments of cytokine production in ED participants (see Table 3 for longitudinal study details and findings) (Nagata et al., 1999; Nova et al., 2002a; Nova et al., 2006; Solis et al., 2002). Only one of the included studies assessed both cross-sectional and longitudinal cytokine production (Nova et al., 2002a).

All included studies assessed cytokine production in participants with AN. Two of these studies reported that their sample consisted exclusively of participants with the restricting type of AN (AN-R) (Nagata et al., 1999; Solis et al., 2002). Two studies reported including participants with AN-R and AN binge-eating/purging type (AN-BP) (Nova et al., 2002a; Nova et al., 2006). Only one study also assessed cytokine production in BN participants (Raymond et al., 2000). There were no studies that measured cytokine production in people with BED or other EDs, aside from AN and BN. ED diagnosis was based on the DSM-IV in 7 studies (American Psychiatric Association, 2000), the DSM-III in 4 studies (American Psychiatric Association, 1980) or the DSM-V (American Psychiatric Association, 2013) in one study. The mean age (reported in 11 studies) and BMI (reported in 7 studies) in AN participants was 17.9 ± 2.8 years and 14.9 ± 2.0 kg/m^2, respectively. Illness duration of AN participants was reported in only two studies (Nagata et al., 1999; Nova et al., 2006) giving a mean illness duration of 2.7 ± 1.9 years. In the one BN study, participants had a mean age of 23.0 ± 6.0 years and BMI or illness duration were not reported (Raymond et al., 2000). The mean age of HCs (reported in 6 studies: Allende et al., 1998; Bessler et al., 1993; Limone et al., 2000; Nova et al., 2002a; Omodei et al., 2015; Raymond et al., 2000) was 20.25 ± 5.9 years and mean BMI (reported in 4 studies: Allende et al., 1998; Limone et al., 2000; Nova et al., 2002a; Omodei et al., 2015) was in the healthy range: 20.8 ± 0.6 kg/m^2. All participants were female, except gender was not reported in one study (Vaisman and Hahn, 1991).

Cytokines assessed in the included studies were granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1α, IL-1β, IL-2, IL-3, IL-3-like activity (LA), IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17α, IL-22, transforming growth factor (TGF)-β and TNF-α. Ten studies measured cytokine production from peripheral blood mononuclear cells (Allende et al., 1998; Bessler et al., 1993; Limone et al., 2000; Nova et al., 2002a; Nova et al., 2006; Omodei et al., 2015; Polack et al., 1993; Raymond et al., 2000; Solis et al., 2002; Vaisman and Hahn, 1991), one from mononuclear cells (Vaisman et al., 1996), and one from whole blood (Nagata et al., 1999). Cytokine measurement methods included enzyme immune-assays (9 studies: Allende et al., 1998; Limone et al., 2000; Nagata et al., 1999; Nova et al., 2002a; Nova et al., 2006; Raymond et al., 2000; Solis et al., 2002; Vaisman et al., 1996; Vaisman and Hahn, 1991), bioassay (1 study: Bessler et al., 1993) or flow cytometric multiplexed bead assays (1 study: Omodei et al., 2015). One study did not provide sufficient details to classify their measurement methods (Polack et al., 1993). All studies assessed stimulated cytokine production, with six studies additionally assessing spontaneous cytokine production (Allende et al., 1998; Bessler et al., 1993; Limone et al., 2000; Nagata et al., 1999; Raymond et al., 2000; Vaisman and Hahn, 1991). Multiple mitogens were used to stimulate cytokine production including phytohaemagglutinin (PHA) (7 studies: Allende et al., 1998; Bessler et al., 1993; Nova et al., 2002a; Nova et al., 2006; Polack et al., 1993; Solis et al., 2002; Vaisman and Hahn, 1991), lipopolysaccharide (LPS) (4 studies: Bessler et al., 1993; Limone et al., 2000; Nagata et al., 1999; Vaisman and Hahn, 1991), autologous (AS) and heterologous serum (HS) (1 study: Omodei et al., 2015), concanavalin A (ConA) (1 study: Raymond et al., 2000), human recombinant granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (1 study: Vaisman et al., 1996), enterotoxin A (EA) (1 study: Allende et al., 1998), and anti-CD3 and anti-CD28 (1 study: Allende et al., 1998).

Reporting of confounding factors (see Table 1). Age matching between ED groups and HCs was reported in 7 of the 9 cross-sectional studies. No studies reported on the smoking status (i.e. smoker vs. non-smoker) of the participants. Six studies reported amenorrhea in all post-menarcheal AN participants. One study measured cytokines in HCs during the early the follicular phase of their menstrual cycle (Limone et al., 2000). Six studies did not report on menstrual status of their participants.

Patients were confirmed medication-free in seven cross-sectional studies and for the duration of one longitudinal study (Nagata et al., 1999). In the three remaining longitudinal studies, participants were administered psychopharmacological medication as part of their inpatient treatment. Medication status was not reported in four studies. Three studies reported that the HCs were free from psychiatric disorders(Allende et al., 1998; Bessler et al., 1993; Nova et al., 2002a), with only one reporting that they used a standardised assessment to ensure this (Allende et al., 1998). An additional study confirmed that the HCs did not have a current or past history of eating disorders and that all participants were free of bipolar illness, psychotic disorders and substance abuse (Raymond et al., 2000). One study reported that n=3 AN participants had a diagnosis of major depressive disorder (Bessler et al., 1993).

With regards to physical health disorders, three studies reported that all participants were free from medical illness and/or were physically healthy. HCs were reported to be free from physical disorders in one study and AN participants were free from medical or associated illnesses in 2 studies. Five studies did not report on the presence or absence of medical illness in their participants(Limone et al., 2000; Solis et al., 2002; Vaisman and Hahn, 1991). In addition, AN participants were reported to be free from infections, infectious diseases and/or acute signs of inflammation in 3 studies.

Table 1. Reporting of confounding factors in the included studies.

Study	Were groups age-matched?	Smoking status of participants	Menstruation	Medication status of patients	Presence of psychiatric disorders	Presence of physical health disorders	Presence of infection or infectious disease
Allende et al. (1998)	Yes	NR	AN: amenorrheic HC: NR	Medication free	HC: free from psychiatric disorders	All: free from medical illness HC: free from neurological conditions	NR
Bessler et al. (1993)	Yes	NR	AN: amenorrheic HC: NR	Medication free	HC: free from psychiatric disorders AN: n=3 diagnosis of MDD	All: physically healthy	NR
Limone et al. (2000)	NR	NR	AN: amenorrheic HC: samples collected in early follicular phase of menstrual cycle.	Medication free	NR	NR	NR
Nagata et al. (1999)	NA	NR	AN-R: amenorrheic HC: NR	Medication free	NR	Free from medical illnesses and no liver or renal dysfunction	Free from infectious disease and no signs of acute inflammation
Nova et al. (2002a)	Yes	NR	AN: amenorrheic HC: NR	Medication free at baseline assessment. Medication given as part of inpatient treatment.	HC: free from psychiatric disorders	HC: free from physical disorders	NR
Nova et al. (2006)	NA	NR	NR	Medication given as part of inpatient treatment.	NR	NR	NR
Omodei et al. (2015)	Yes	NR	NR	NR	NR	NR	AN: free from infectious disease
Polack et al. (1993)	Yes	NR	NR	Medication free	NR	AN: free from associated illnesses	NR
Raymond et al. (2000)	NR	NR	NR	NR	All: free from bipolar and psychotic disorders, and substance abuse HC: free from EDs	All: free from medical illness	NR
Solis et al. (2002)	NA	NR	NR	Medication given as part of inpatient treatment.	NR	NR	NR
Vaisman and Hahn (1991)	Yes	NR	NR	NR	NR	NR	NR
Vaisman et al. (1996)	Yes	NR	AN: post-menarchal participants amenorrheic HC: NR	NR	NR	NR	AN: free from infection

Abbreviations: NR = not reported; NA = not applicable; HC = healthy control; AN = anorexia nervosa; MDD = major depressive disorder; ED = eating disorder

3.3. Study Findings

3.3.1. Cross-sectional studies in anorexia nervosa (see Table 2)

Spontaneous cytokine production. Unstimulated cytokine production was measured in 5 studies (Allende et al., 1998; Bessler et al., 1993; Limone et al., 2000; Raymond et al., 2000; Vaisman and Hahn, 1991). No differences between AN participants and HCs were observed in production of IFN-γ, IL-1, IL-1α, IL-3LA, IL-6, and TGF-β. One study found TNF-α production to be higher in AN participants to HCs (Vaisman and Hahn, 1991), however, two studies found no significant difference between the groups (Limone et al., 2000; Raymond et al., 2000). Similarly, IL-1β production was elevated in AN compared to HC participants in one study (Allende et al., 1998), but did not differ between groups in another study (Limone et al., 2000).

Stimulated cytokine production. Nine studies measured stimulated cytokine production using methods. Findings related to stimulated IFN-γ production were mixed: two studies found no difference between AN participants and HCs (Nova et al., 2002a; Omodei et al., 2015), one study found elevated IFN-γ production (Raymond et al., 2000) and one found reduced production (Polack et al., 1993) in AN participants compared to HCs.

AN participants and HCs did not differ in IL-1 production in two studies (Bessler et al., 1993; Vaisman et al., 1996). Two studies found participants with AN to have higher stimulated IL-1β production than HCs (EA and anti-CD3 only, Allende et al., 1998; Nova et al., 2002a) and two studies found no difference between AN and HC groups (Limone et al., 2000; Omodei et al., 2015). Three studies identified no difference in IL-2 stimulated production between AN and HC groups (Allende et al., 1998; Nova et al., 2002a; Omodei et al., 2015). One study found lower production in AN compared to HCs (Bessler et al., 1993). Stimulated IL-6 production did not differ between AN and HC participants in 3 studies (AS only, Omodei et al., 2015; Raymond et al., 2000; Vaisman et al., 1996). One study found lower levels of production in AN participants (Nova et al., 2002a) and one found higher (HS only, Omodei et al., 2015) compared to HCs.

The majority of studies found no difference in stimulated TNF-α production between AN and HC participants (Limone et al., 2000; Omodei et al., 2015; Raymond et al., 2000; Vaisman and Hahn, 1991); however, one study found lower stimulated TNF-α production in AN participants compared to HCs (Nova et al., 2002a).

Stimulated production of several other cytokines were assessed in only one study, including IL-1α, IL-4, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-17α, IL-22 and TGF-β and were found to not differ between AN participants and HCs (Omodei et al., 2015; Raymond et al., 2000). GM-CSF (Vaisman et al., 1996) and IL-3 (Allende et al., 1998) stimulated production were also reported in only one study and were found to be reduced in AN compared to HC participants.

3.3.2. Cross-sectional studies in bulimia nervosa (see Table 2)

In the only study to assess cytokine production in people with BN, both unstimulated and ConA-stimulated production of IFN-γ, IL-1α, IL-6 and TNF-α did not significantly differ between BN participants and HCs.

Table 2. Study characteristics and findings from *in vitro* cross-sectional studies.

Study	N	Sample	Mean age ± SD (years)	Mean BMI ± SD (kg/m^2)	Diagnostic criteria	Parameters of interest	Mitogen used	Measurement method	Findings		
									Spontaneous cytokine production	Stimulated cytokine production	Additional findings
Studies in anorexia nervosa											
Allende et al. (1998)	54	Female AN (n=40) Female HC (n=14)	16.8 ± 2.3 15.7 ± 1.2	17.8 ± 1.6 20.6 ± 1.5	DSM-IV	IL-1β, IL-2, IL-3	EA 1 ng/ml PHA 1:100 final dilution Anti-CD3 12.5 ng/ml Anti-CD28 50 ng/ml	PBMCs - ELISA	IL-1β: AN>HC	IL-1β: AN>HC (EA, anti-CD3 only); AN=HC (anti-CD28, PHA only) IL-2: AN=HC IL-3: AN<HC (anti-CD3, anti-CD28 only)	
Bessler et al. (1993)	20	Female AN (n=10) Female HC (n=10)	17.6 ± 1.6 17.8 ± 0.6	NR NR	DSM-III	IL-1, IL-2, IL-3-LA	IL-1: LPS 10 µg/mL IL-2: PHA 1 µg/mL	PBMCs - bioassay	IL-1, IL-3LA: AN=HC	IL-1: AN=HC IL-2: AN<HC	
Limone et al. (2000)	26	Female AN (n=13) Female HC (n=13)	18.0 ± 2.1 19.0 ± 1.8	12.9 ± 1.7 21.7 ± 1.8	DSM-IV	IL-1β, TNF-α	LPS 10 µg/mL	PBMCs - enzyme immunoassay	AN=HC	AN=HC	In AN, unstimulated IL-1β production positively correlated with serum cortisol levels.
Nova et al. (2002a)	72	Female AN (n=37) Female HC (n=35)	15.8 ± 1.5 15.4 ± 1.3	15.4 ± 1.4 20.6 ± 2.7	DSM-IV	IFN-γ, IL-2, TNF-α, IL-6, IL-1β	PHA 7 pg/ml	PBMCs - ELISA		IFN-γ, IL-2: AN=HC TNF-α, IL-6: AN<HC IL-1β: AN>HC	Illness duration correlated with IL-1β (negative) and IFN-γ (positive) production.
Omodei et al. (2015)	35	Female AN (n=15)	19.7 ± 3.8 22.6 ± 3.7	16.0 ± 1.6	DSM-V	TNF-α, IFN-γ, IL-1β, IL-2,	Autologous serum (AS)	PBMCs - Human Th1/Th2/Th9/Th17/Th22 13plex kit FlowCytomix		IL-6 (HS only): AN>HC	

		Female HC (n=20)		20.4 ± 3.1		IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17α, IL-22	Heterologous serum (HS)			TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6 (AS only), IL-9, IL-10, IL-12p70, IL-13, IL-17α, IL-22: AN=HC	
Polack et al. (1993)	60	Female AN (n=30) Female HC (n=30)	16.5 ± 2.1 NR	NR NR	DSM-III	IFN-γ	PHA 10 µg/mL	PBMC – Measurement of antiviral activity of IFN-γ		AN<HC	After 18-months of treatment, in 2/4 AN participants, IFN-γ production AN=HC.
Raymond et al. (2000)	42	Female AN (n=19) Female HC (n=23)	23.0 ± 8.0 31.0 ± 9.0	NR NR	DSM-IV	IL-6, IFN-γ, IL-1α, TNF-α, TGF-β	ConA 12.5 mg/ml	PBMC - ELISA	AN=HC	IFN-γ: AN>HC IL-6, IL-1α, TNF-α, TGF-β: AN=HC	
Vaisman and Hahn (1991)	23	AN (n=7) HC (n=16)	15.1 ± 1.4 NR	NR NR	DSM-III	TNF-α	LPS 10 µg/mL PHA 20 µg/mL	PBMC - ELISA	AN>HC	AN=HC	During refeeding, spontaneous and induced TNF-α production gradually decreased.
Vaisman et al. (1996)	17	Female AN (n=10) Female HC (n=7)	15.8 ± 1.6 NR	NR NR	DSM-III	IL-1, IL-3, IL-6, GM-CSF, TNF	rhGM-CSF 10 ng/mL	MNC - ELISA		IL-1, IL-6: AN=HC GM-CSF: AN<HC IL-3, TNF: ND	
Studies in bulimia nervosa											
Raymond et al. (2000)	39	Female BN (n=16) Female HC (n=23)	23.0 ± 6.0 31.0 ± 9.0	NR NR		IL-6, IFN-γ, IL-1α, TNF-α, TGF-β	ConA 12.5 mg/ml	PBMC - ELISA	BN=HC	BN=HC	

Abbreviations: N = number of subjects; SD = standard deviation; BMI = body mass index; AN = anorexia nervosa; HC = healthy control; DSM = Diagnostic and Statistical Manual of Mental Disorders; IL = interleukin; EA = enterotoxin A; PHA = phytohaemagglutinin; PBMC = peripheral blood mononuclear cell; ELISA = enzyme-linked immunosorbent assay; NR = not reported; LA = like activity; LPS = lipopolysaccharide; TNF = tumor necrosis factor; IFN = interferon; TGF = transforming growth factor; ConA = concanavalin A; GM-CSF = granulocyte-macrophage colony-stimulating factor; rhGM-CSF = human recombinant granulocyte-macrophage colony-stimulating factor; MNC = mononuclear cells; BN = bulimia nervosa.

3.3.3. Longitudinal studies in anorexia nervosa (see Table 3)

The four studies measuring cytokine production longitudinally differed significantly in intervention type. Firstly, two assessed cytokine changes in response to inpatient treatment (Nagata et al., 1999; Nova et al., 2002a). Nagata et al. (1999) found that LPS-stimulated production of IFN-γ, IL-1α, IL-1β, IL-6 and TNF-α increased as a function of weight gain, whereas G-CSF production remained stable at all time points. In contrast, Nova et al. (2002a) found that PHA-stimulated production of IFN-γ, IL-1β, IL-2, IL-6 and TNF-α did not differ between admission and discharge from inpatient treatment.

Secondly, the remaining two studies conducted randomised controlled trials assessing the impact of dairy product consumption (yoghurt vs. milk) on cytokine production during inpatient treatment (Nova et al., 2006; Solis et al., 2002). Both studies found that 10-weeks of daily yoghurt (containing *lactobacillus delbrueckii* subsp. *bulgaricus* and *streptococcus thermophilus)* consumption increased PHA-stimulated IFN-γ production. In response to milk consumption, IFN-γ production reduced in both studies. However, if participants had firstly engaged in yoghurt consumption for 10-weeks, milk had no effect on IFN-γ production (Solis et al., 2002). Other cytokines also measured (IL-1β, IL-2, IL-6, TNF-α) remained stable throughout the intervention (Nova et al., 2006). It is important to note that weight gain in AN participants was observed in both studies, regardless of dietary intervention.

3.3.3. Longitudinal studies in bulimia nervosa

No studies investigated cytokine production longitudinally using *in vitro* methods in BN.

Table 3. Study characteristics and findings from *in vitro* longitudinal studies.

Study	N	Sample	Mean age ± SD (years)	Mean BMI ± SD (kg/m^2)	Diagnostic criteria	Intervention	Assessment time points	Parameters of interest	Mitogen used	Measurement method	Findings		
											Spontaneous cytokine production	Stimulated cytokine production	Additional findings
Studies in anorexia nervosa													
Nagata et al. (1999)	17	Female AN-R	22.7 ± 6.1	11.6 ± 1.3	DSM-IV	Inpatient treatment	T1: At admission (<60% SBW) T2: At 65% SBW T3: At 75% SBW	IL-1α, IL-1β, IL-2, Il-6 TNF-α, G-CSF, IFN-γ	LPS 10 µg/mL	Whole blood - ELISA	ND	IFN-γ, IL-1α, IL-1β, Il-6, TNF-α: T0>T1, T0>T2 G-CSF: T0=T1=T2 IL-2: ND	No significant correlations between cytokine production and demographic or clinical variables.
Nova et al. (2002a)	37	Female AN	15.8 ± 1.5	15.4 ± 1.4	DSM-IV	Inpatient multidisciplinary treatment	T0: At admission T1: At discharge	IFN-γ, IL-2, TNF-α, IL-6, IL-1β	PHA 7 pg/ml	PBMCs - ELISA		T0=T1	IFN-γ, IL-6, TNF-α: T1<HC IL-2: T1=HC IL-1β: T1>HC
Nova et al. (2006)	30	Female AN	16.1 ± 1.9	15.1 ± 1.7	DSM-IV	AN-m (n=14): consumed 400 ml/day of milk for 10 weeks AN-y (n=16): consumed 375 g/day of yoghurt for 10 weeks	T0: At admission T1: After 6 weeks of dairy product consumption T2: After 10 weeks of dairy product	IFN-γ, IL-2, IL-6, IL-1β, TNF-α	PHA 10 µg/mL	PBMCs - ELISA		IFN-γ: AN-m T0>T2, AN-y T0<T2 IL-2, IL-6, IL-1β, TNF-α: T0=T2	

							consumption				
Solis et al. (2002)	27	Female AN-R	NR	15.4 ± 1.6	DSM-IV	AN1 (n=16): consumed 375 g/day of yoghurt for 10 weeks, followed by 450 g/day of milk for 10 weeks AN2 (n=11): consumed 450 g/day of milk for 10 weeks, followed by 375 g/day of yoghurt for 10 weeks	T0: Baseline T1: Week 6 T2: Week 10 T3: Week 16 T4: Week 20	IFN-γ	PHA	PBMCs - ELISA	AN1: T0<T2, T0<T4, T2=T4 AN2: T0>T2, T0=T4, T2<T4

Abbreviations: N = number of subjects; SD = standard deviation; BMI = body mass index; AN-R = anorexia nervosa restricting type; HC = healthy control; DSM = Diagnostic and Statistical Manual of Mental Disorders; T = time point; IL = interleukin; TNF = tumor necrosis factor; G-CSF = granulocyte-colony stimulating factor; IFN = interferon; LPS = lipopolysaccharide; ELISA = enzyme-linked immunosorbent assay; ND = not detected; AN = anorexia nervosa; PHA = phytohaemagglutinin; PBMC = peripheral blood mononuclear cell; NR = not reported

4. Discussion

This systematic review summarises and integrates the existing data on spontaneous and stimulated cytokine production across EDs. All studies reported on findings in AN, with only one study also measuring cytokine production in people with BN (Raymond et al., 2000). No studies reported on cytokine production in BED or other EDs. While a broad range of cytokines were measured, few cytokines were measured across multiple studies. Limited assessments in BN and of certain cytokines across multiple studies, in combination with the heterogeneity in the study samples, design, and methodologies, precludes definitive conclusions on the relationship between cytokine production and EDs.

4.1. Findings in anorexia nervosa

Cross-sectional studies. Findings were inconsistent across studies in relation to spontaneous and stimulated production of the following cytokines: IFN-γ, IL-1β, IL-2, Il-6 and TNF-α. Most studies found no difference between AN participants and HCs, however, a few studies identified reductions and/or elevations in the production of these cytokines in people with AN compared to HCs. Other cytokines (IL-1α, IL-3, GM-CSF, TGF-β) were only assessed in individual studies and therefore, conclusions cannot be drawn. Across two studies IL-1, a potent anorexigenic pro-inflammatory cytokine (Wong and Pinkney, 2004), was found to consistently not differ between people with AN and HCs (Bessler et al., 1993; Vaisman et al., 1996).

Previous studies have identified similar inconsistent findings in circulating concentrations of these cytokines (e.g., Allende et al., 1998; Brambilla et al., 2001; Nogueira et al., 2010; Ostrowska et al., 2015). Although, when taken together, recent meta-analyses found elevated levels of the pro-inflammatory cytokines IL-6 and TNF-α in AN participants compared to HCs (Dalton et al., 2018; Solmi et al., 2015). However, it is important to note that circulating levels of cytokines measured *in vivo* in patients with EDs are influenced by ED-associated behaviours and subsequent biological processes. In AN, for example, leptin, cortisol and ghrelin are altered as a consequence of self-starvation (Himmerich et al., 2010) and these hormones have been shown to have effects on the immune system (Himmerich and Sheldrick, 2010). Therefore, an *in vitro* approach, using purified immune cells, may be more suitable to examine the function of the immune system without the influence of the metabolic changes which are a consequence of the ED. In order to investigate whether the immune system may be causally involved in the development of an ED, it seems sensible to perform immunological studies, where the metabolic implications of the ED are neutralised as much as possible.

Research in other psychiatric disorders, including depression, schizophrenia and post-traumatic stress disorder, has found that symptom severity and other clinical characteristics (e.g., illness duration, age of onset) are associated with cytokine levels (Dunjic-Kostic et al., 2013a; Dunjic-Kostic et al., 2013b; Gill et al., 2008). In one included study, illness duration was found to negatively correlate with IL-1β production and positively with IFN-γ (Nova et al., 2002a). However, in Nagata et al. (1999) clinical variables did not significantly correlate with cytokine production. More studies in EDs are needed to examine the relationship between cytokines and clinical characteristics.

There are several methodological factors that may account for the conflicting findings, including small samples, diverging experimental designs and cytokine measurement methods, and confounding factors. With regards to the measurement method, a variety of mitogens, cell types and assays were used. *In vitro* cytokine production can be differentially affected depending on the mitogen used (Ai et al., 2013; Allende et al., 1998), which given the variety of stimulants used across the include studies, may limit the comparability of studies and also may account for the mixed results. In addition, the procedures used to extract PBMCs from the rest of the blood may wash out important plasma molecules and thus disrupt molecular networks between immune cells; therefore, data on cytokine production derived from the use of PBMCs outside the blood environment may not reflect how PBMCs would respond *in vivo* (Ai et al., 2013). Ultimately, due to variation in experimental design and a lack of standard values, there is a difficulty in interpreting *in vitro* study results. In order to implement these normal limits, the experimental design needs to become standardised. This could

involve standardised blood drawing procedures, a standardised method of immunological stimulation and incubation of the cells, and a generally accepted method for the measurement of cytokines.

Our review of confounding variables in the included studies (see Table 1) highlighted that several important pre-analytical factors had not been incorporated into the design and/or analysis of the studies, including smoking, medication status, and presence of concurrent physical and mental illnesses (Dugué et al., 1996). For example, none of the included studies reported participant's smoking status even though smoking is known to alter cytokine production (Petrescu et al., 2010) and to also influence the gut microbiota (Benjamin et al., 2012) which, in turn, modulates cytokine signalling (Haase et al., 2018). Additionally, in the majority of studies, co-morbid psychiatric conditions in the AN participants were not assessed, despite alterations in cytokine levels being identified in disorders such as depression (Lichtblau et al., 2013) and anxiety (Baldwin et al., 2017). Similarly, only one study reported using a standardised assessment to ensure the absence of psychiatric disorders in their HC group (Allende et al., 1998). Therefore, we cannot rule out the possibility that psychiatric conditions contributed to the results observed in the included studies. Furthermore, not all studies screened their participants for autoimmune and autoinflammatory diseases. Given that excessive, reduced, or aberrant cytokine responses contribute to the pathogenesis of these illnesses (Moudgil and Choubey, 2011) and autoimmune and anti-inflammatory diseases have been shown to be associated with an increased risk for EDs (Raevuori et al., 2014; Zerwas et al., 2017), future studies need to ensure all participants are assessed for the presence of these diseases.

Longitudinal studies. Contradictory findings between two longitudinal studies assessing cytokine production in inpatient treatment settings were found: Nagata et al. (1999) found that stimulated production of IFN-γ, IL-1α, IL-1β, IL-6 and TNF-α increased and Nova et al. (2002a) found that production of IFN-γ, IL-1β, IL-2, IL-6 and TNF-α remained stable over time. The final assessment point was different in each of these studies, with one focusing on meeting certain weight targets, specifically when 75% of SBW was reached (Nagata et al., 1999), and the other basing the timing of the follow-up assessment on discharge from the inpatient hospital, for which both weight recovery and psychological evaluation were taken into consideration (Nova et al., 2002a). Variance in the inpatient treatment received by participants may have also contributed to the observed findings: in Nagata et al. (1999) treatment was focused around refeeding with some cognitive-behavioural treatment and no medication, whereas in Nova et al. (2002a) psychopharmacological medication was additionally provided. Psychotropic medication (e.g., antidepressants, antipsychotics) has been shown to influence cytokine production (Himmerich et al., 2011; Munzer et al., 2013) and therefore, may have contributed to the results observed in Nova et al. (2002a).

Both dietary intervention trials found that daily yoghurt consumption for 10-weeks increased IFN-γ production (Nova et al., 2006; Solis et al., 2002). However, other cytokines (IL-1β, IL-2, IL-6, TNF-α) were not found to change as a result of the intervention (Nova et al., 2006). The authors suggest that potential beneficial effects of increased IFN-γ on the immune response could be presumed, given that previous studies have indicated inhibition of PHA-induced IFN-γ production in people with AN compared to HCs (Polack et al., 1993; Schattner et al., 1990). However, these studies are not capable of proving that an increase in IFN-γ would ameliorate immune defence in AN patients. The authors are also unclear on how cytokine production is modulated by yoghurt consumption. As the lactic acid bacteria contained in the yoghurt used in the interventions (*lactobacillus delbrueckii* subsp. *bulgaricus* and *streptococcus thermophilus)* has been shown to have little to no viability in the human gut and a limited ability to influence the composition of the gut microbiota (Pei et al., 2017), more research is needed to determine the mechanisms by which yoghurt influences cytokine production. It is important to consider that the translation of *in vitro* findings to *in vivo* implications is difficult, therefore, we cannot be sure that these studies are proof-of-concept that it is possible to modify cytokine production in people with AN through a simple dietary intervention. Future research should focus on whether these and other interventions, particularly those currently being researched in the treatment of EDs (e.g., psychological therapy, brain stimulation, cognitive bias modification), have the ability to modulate concentrations of circulating cytokines.

None of the longitudinal studies reported standardised assessments of ED symptoms at the various time points cytokine production was assessed. This therefore precludes any examination of whether longitudinal changes in cytokine production are associated with the psychological symptoms of the ED or weight gain. Future research should ensure assessment of ED severity at all time points to allow for examination of this and determine whether cytokines are a marker of treatment response.

4.2. Findings in bulimia nervosa

Only one study measured cytokine production in people with BN, identifying no differences between BN participants and HCs in spontaneous or stimulated production of IFN-γ, IL-1α, IL-6 and TNF-α (Raymond et al., 2000). These findings are similar to that observed in two studies that measured cytokine concentrations *in vivo* (Brambilla et al., 1998; Monteleone et al., 1999). Although concentrations of IL-6 and TNF-α have also been found to be elevated in people with BN, compared to HCs in two studies (Ahren-Moonga et al., 2011; Nakai et al., 2000). Binge eating behaviours (as assessed by a range of questionnaires) have also been shown to be significantly and positively associated with TNF-α concentrations in adolescents (Lofrano-Prado et al., 2011). Ultimately few studies have considered the role cytokines play in BN and also binge eating behaviours more specifically. This highlights the need for future studies to measure cytokines across EDs presenting with binge-eating behaviours i.e. BN, BED and AN-BP.

4.3. Conclusions

Given the contradictory findings presented in the current review, it is unclear whether ED participants differ in cytokine production in comparison to HCs. This variation in findings could be accounted for by various methodological issues highlighted in this review, such as limited reporting and consideration of confounding factors in the measurement of cytokines, the variability in equipment and methods used to measure cytokines, and small sample sizes. Given the identified methodological heterogeneity and shortcomings of the included and evaluated *in vitro* studies, it seems unreasonable to draw far-reaching conclusions from their results. Only one study assessed cytokine production in BN (Raymond et al., 2000) and no studies considered cytokine production in other EDs, such as binge eating disorder. This highlights the need for more research in these patient groups.

The findings of the longitudinal studies assessing cytokines production in AN during inpatient treatment were also mixed (Nagata et al., 1999; Nova et al., 2002a). This could be due to confounding factors of medication, weight gain, and variability in treatment. However, two longitudinal studies found that IFN-γ production increased in response to a dietary intervention (Nova et al., 2006; Solis et al., 2002). Therefore, evidence from these studies indicates that it may be possible to influence *in vitro* IFN-γ production in people with AN, however, more evidence is needed to support such a conclusion.

Future research would benefit from measuring cytokine production alongside psychological assessments over the treatment course to gain a clearer understanding of how cytokines are related to treatment response and their association with both psychological and weight changes. Furthermore, longer-term research studies may help to elucidate the role of cytokines in EDs and to determine whether augmented cytokine production are state or trait marker of EDs.

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